Food and Chemical Toxicology 97 (2016) 265–276

Contents lists available at ScienceDirect

Food and Chemical Toxicology

journal homepage: www.elsevier.com/locate/foodchemtox

Enhanced cytotoxic and apoptosis inducing activity of lycopene oxidation products in different cancer cell lines

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ARTICLE INFO

Article history:
Received 17 February 2016
Received in revised form
10 September 2016
Accepted 12 September 2016
Available online 13 September 2016

Keywords:
Lycopene oxidation products
Cytotoxicity
Apoptosis
Reactive oxygen species
Free radicals
UPLC-MS

ABSTRACT

Currently, upon understanding the metabolomics of carotenoids, it is important to address the key role of carotenoid derived products. In this regard, aim of the study was to elucidate and explore the role of lycopene (LYC) oxidative products generated through autoxidation (AOL) or chemical ($KMnO_4$) oxidation (COL) against proliferation of selected cancer cells. Preliminary, we investigated the effect of LYC on cell viability of various cancer cell lines (PC-3, MCF-7, A431, $HepG_2$, HeLa and A549). Based on the results of LYC treatment on cell cytotoxicity levels, MCF-7, PC-3 and HeLa cell lines were further tested with AOL and COL products. The decreased cell viability with depleted GSH and increased MDA levels were observed when treated with COL products than control, LYC and AOL. In addition, COL products increased ROS levels and percent apoptosis. The typical morphological changes and nuclear condensations showed that COL products have anti-proliferation and apoptosis inducing activity. Based on results, we hypothesized that ROS generation by LYC oxidation products may be one of intermediate step involved in apoptosis. The redox status and therapeutic approach of COL products in modulating ROS and induction of apoptosis in cancer cells were reported for the first time, to our knowledge. To conclude, COL products involves in cancer growth inhibition efficiently than intact LYC and AOL. Hence, there is a great potential for synthesizing or producing such carotenoid oxidation products to augment cancer complication.

1. Introduction

Epidemiological studies have shown that consumption of carotenoids rich food are associated with a reduced risk of cancer and other chronic diseases (Rao and Agarwal, 2000; Giovannucci et al., 2002; Etminan et al., 2004; Talvas et al., 2010). Among carotenoids, LYC is one of the highly recognised bioactive compound involved in inhibition of prostate and other cancers by various mechanisms (Nahum et al., 2001; Livny et al., 2002; Liu et al., 2003; Tang et al., 2005; Herzog et al., 2005). The continuous progress in carotenoids research demonstrates that the biological role of them is attributed to identification of their metabolites or oxidative products (Wang, 2012). In general, carotenoid metabolites are reported to be involved in chemoprevention of cancers. The conversion and biological significance of β-carotene oxidative cleavage products (retinal, retinoic acid and apo-carotenoids) are well documented in mammals (Eroglu and Harrison, 2013). Similarly, some progress has been made on lutein oxidation products and their biofunctional property (Khachik et al., 1997; Lakshminarayana et al., 2013; Nidhi et al., 2015). However, information related to metabolism of non-provitamin A carotenoids like LYC are yet to be described in detail. Previously, Khachik et al. (1997) identified carotenoid metabolites in human milk and serum. Subsequently, Kim et al. (2001) have shown the formation of LYC oxidation products in vitro and proposed the presence of similar compounds in vivo samples due to autoxidation. Later, Caris-Veyrat et al. (2003) identified apo-lycopenals and apo-lycopenones derived from oxidation of LYC with potassium permanganate and by atmospheric oxygen catalyzed by a metalloporphyrin. Further, central and eccentric cleavages of LYC in the presence of soy lipoxygenase were identified in

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http://dx.doi.org/10.1016/j.fct.2016.09.016
0278-6915/

Abbreviations

LYC	Lycopene
AOL	Autoxidation lycopene
COL	Chemical oxidation lycopene
$KMnO_4$	Potassium per manganate
PC-3	Human prostate cancer cell line
MCF-7	Human breast adenocarcinoma cell line
A431	Human epidermoid carcinoma cell line
$HepG_2$	Human hepatocelluar carcinoma cell line
HeLa	Human cervical cancer cell line
A549	Human alveolar epithelial cell line
ROS	Reactive oxygen species
DCHF-DA	Dichloro-dihydro-fluorescein diacetate
DAPI,4′	6-diamidino-2-phenylindole
BHT	Butylated hydroxytoluene
DTNB	5-5′-dithiobis [2-nitrobenzoic acid]
MDA	Malondialdehyde
EDTA	Ethylenediaminetetraacetic acid
NADPH	Nicotinamide adenine dinucleotide phosphate
DMEM	Dulbecco's minimal essential medium
MEM	Minimum essential medium
FBS	Fetal bovine serum
MTT	3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide
PBS	Phosphate buffer saline
FITC	Fluorescein isothiocyanate
CTAB	Cetyltrimethylammonium bromide
PVDF	Polyvinylidene fluoride
DCM	Dichloromethane
UPLC	Ultra performance liquid chromatography
PDA	Photodiode array
APCI	Atmospheric-pressure chemical ionization
H_2O_2	Hydrogen peroxide
AO/EB	Acridine orange/Ethidium bromide
HOCl	Hypochlorous acid

rat intestinal mucosa (Ferreira et al., 2004). Gajic et al. (2006) have determined the existence of apo-8′- lycopenal, apo-12′-lycopenal and other polar metabolites of LYC in rats. Likewise, Kopec et al. (2010) have shown the presence of a series of apo-lycopenals in fruits, vegetables and human plasma and anticipated the formation may be due to enzymatic and chemical oxidative cleavage of LYC. The discovery of various oxidation products or metabolites of carotenoids either in vitro or in vivo, have questioned the active role of them compared to intact carotenoid molecules in ameliorating various diseases. However, little is known about metabolites or oxidative products of LYC on health benefits. Nara et al. (2001) have demonstrated that autoxidation mixtures of LYC inhibited the HL-60 cell growth effectively than LYC. Similarly, Zhang et al. (2003) identified a cleavage product of LYC (E, E, E)-4-methyl-8-oxo-2, 4, 6-nonatrienal, and evaluated its apoptosis-inducing activity in HL-60 cells. Consequently, Aust et al. (2003) reported the role of LYC degraded products in enhancing cell communication and cell signalling. Generally, the process of apoptosis can be initiated by various physiological and physico-chemical stimuli like oxidants, ionizing radiation and chemotherapeutic agents. Notably, carotenoids are shown to be an effective antioxidant by scavenging certain reactive oxygen species (ROS), especially peroxyl radicals and singlet oxygen at low oxygen tension (Burton and Ingold, 1984; Halliwell, 1999). However, clinical trials of β-carotene supplements on the incidence of cancer could be non-protective or detrimental in smokers (Omenn et al., 1996). Therefore, the antioxidant and anti-carcinogenic properties of carotenoids need to be re-evaluated at different situations like higher oxygen tension and ROS production. It has been suggested that β-carotene or its degradation products may be pro-oxidant or pro-carcinogenic (Wang and Robert, 1999). Increased generation of reactive oxygen species (ROS) and an altered redox status have been observed in cancer cells, and recent studies suggest that this biochemical property of cancer cells can be exploited for therapeutic benefits (Prasad et al., 2006; Cui et al., 2007). Few studies have correlated that, β-carotene induces apoptosis through ROS intermediates (Prasad et al., 2006; Cui et al., 2007). LYC is another important non-provitamin A carotenoid with same molecular mass, chemical formula as β-carotene and differs structurally by open polyene chain lacking with β-ionone ring. Hydrocarbon carotenoids like β-carotene and LYC are known for their superior antioxidant against singlet and other reactive oxygen species, however, studies have shown the moderate action of these carotenoids on various cancer cell lines in contrast to the reports of in vivo studies. Previously, Teodoro et al. (2012) demonstrated that lycopene inhibits cell proliferation and increased apoptosis effectively in breast, colon and prostate cancer cells among eight different cancer cell lines. Further, they suggested effect of lycopene may vary with cellular type, time and dose. Recently, studies of others including our laboratory have an opinion that cellular effects of carotenoids are mediated through their derivatives formed either by chemical oxidation or by enzymatic cleavage in vivo system (Sharoni et al., 2012; Lakshminarayana et al., 2008, 2013). Hence, elucidation of LYC oxidation products and evaluation of their biological significance is warranted. With this back ground, the aim of the present study was to investigate the role of LYC oxidative products on cell viability, oxidation status, ROS generation, their impact on cell cycle progression and apoptosis. Initially, the effect of LYC on cytotoxicity of various cancer cell lines (PC-3, MCF-7, A431, $HepG_2$, HeLa and A549) was evaluated. Based on this, cell lines PC-3, MCF-7 and HeLa with maximum growth inhibition by LYC were chosen to understand the role of LYC oxidation products, expecting that outcome of the study will have the potential to treat cancer and other health associated problems.

2. Materials and methods

2.1. Materials

(all-E)-Lycopene (>90%), Propidium iodide, toluene, potassium permanganate, Poly-D-lysine, ethidium bromide, acridine orange, dichloro-dihydro-fluorescein diacetate (DCHF-DA) dye, 4,6-diamidino-2-phenylindole dilactate (DAPI) and butylated hydroxytoluene (BHT), tetrahydrofuran (stabilized with BHT) were purchased from Sigma-Aldrich (St Louis, MO, USA). Glutathione, glutathione reductase, 5, 5-dithiobis (2-nitrobenzoic acid) (DTNB), NADPH, EDTA were purchased from Sisco Research Laboratories (Mumbai, India). Acetone, hexane, methanol, acetonitrile and tetrahydrofuran of HPLC- grade solvents, PVDF syringe filters (0.45 μm) were procured from Merck (Mumbai, India). Dulbecco's minimal essential medium (DMEM), Minimum essential medium (MEM), Ham's F-12 media, fetal bovine serum (FBS), 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), antibiotic antimycotic solution, calcium magnesium free phosphate

buffer saline (PBS), all other cell culture consumables were purchased from Hi-Media Chemical Laboratories (Mumbai, India). FITC Annexin-V apoptosis detection kit was purchased from BD pharmingen (BD Bioscience, San Diego, CA). All other chemicals and solvents of analytical grades were purchased from Sisco Research Laboratories (Mumbai, India).

2.2. Isolation of lycopene from the tomato

LYC was extracted from ripened stage tomatoes (Indian hybrid) as per our method Arathi et al. (2015a). The peak identity, absorption maxima (λ_{max}), and characteristics UV–Visible spectra of LYC was confirmed by UPLC-PDA-MS ($APCI^{+ve}$). Extraction and preparation of LYC samples and standards were carried out under dim yellow light at 4 °C to prevent isomerization and degradation.

2.3. Preparation of lycopene autoxidation products

For autoxidation mixtures (AOL), LYC (5 mg/5 mL) was dissolved in toluene and incubated in a water bath at 37 °C for 24 h under atmospheric oxygen condition with regular shaking (100 rpm). The autoxidised products of LYC were extracted as per the established method (Arathi et al., 2015a) and verified by using characteristic absorption spectra obtained by UPLC (Emenhiser et al., 1995).

2.4. UPLC-MS conditions for LYC and AOL analysis

Qualitative analysis of LYC and AOL was done by using Waters Xevo TQD mass spectrometer interfaced with the ACQUITY UPLC® system via an APCI source operated in positive ion mode. An aliquot (2 μL) of LYC sample containing ~40 μg/mL was injected onto the CSH Phenyl-hexyl column (100 × 2.1 mm; 1.7 μm). Mobile phase contained 10 mM ammonium acetate in Milli-Q water (A) and 5% of THF in acetonitrile (B) with the flow rate of 0.4 mL/min and was monitored at 471 nm by using PDA detector. The gradient condition of mobile phase was maintained as follows: 25% of A and 75% of B solvents was fixed initially, then increased solvent B to 85% in 3 min, 90% in 8 min and returned to 75% in 8.2 min. Mass spectrometry was conditioned by following parameters: corona voltage 0.9 kV, cone 35 V, RF 2.50 V, extractor 3.00 V, source temperature 150 °C, probe temperature 450 °C, cone gas flow 50 L/hour, desolvation gas flow 900 L/hour. Mass spectra of LYC and AOL were acquired with an *m/z* 0–1000 scan range. The MS identity of standard (all-E)-LYC was compared with the purified LYC sample. Data were processed with Mass Lynx 4.1 software (Waters, USA).

2.5. Lycopene oxidation by $KMnO_4$

LYC was subjected to oxidation by using $KMnO_4$ as per the procedure of Catherine Caris-Veyrat et al. (2003) with slight modification. In brief, LYC (30 mg) and cetyltrimethylammonium bromide (6 mg) were dissolved in 30 mL DCM/toluene (1:1 ratio) and made 6 aliquots (5 mg/5 mL) separately for degradation study. The oxidation of LYC was initiated by adding 1.5 mL of aqueous solution of $KMnO_4$ stock (135 mg in 9 mL water) to the LYC sample. The reaction mixture was stirred on a magnetic stirrer and incubated for different time points (5, 10, 15, 20, 30 & 40 min) at room temperature (26 ± 2 °C). Then, separation of the organic phase was done by repeated washing with double distilled water (10 mL). The organic solvent phase of LYC oxidised sample was filtered through a PVDF filter (0.45 μm) and dried on sodium sulphate under nitrogen environment. The residue of LYC oxidised mixture was re-dissolved in petroleum ether and filtered again with PVDF filter to remove the $KMnO_4$ remnant. Oxidative products obtained were analysed by MS - APCI $^{+ve}$ mode. The degradation time point ODT_{100} and degradation half ODT_{50} of LYC incubated with $KMnO_4$ was calculated, and the optimal ODT_{50} was preferred for further cell culture treatments. The aliquots of LYC and its oxidative products were sealed under nitrogen environment and used for cell culture treatments or stored in -80 °C in amber vials.

2.6. MS conditions and analysis of lycopene oxidation products

MS analysis of LYC and COL products was performed on the Thermo Scientific, LC-MS system coupled to an Ion Trap mass spectrometer (LCQ Deca XP Max). The capillary and vaporizer temperatures were set at 298 °C and 300 °C. The corona discharge current was (5 μA), Entrance lens (34.6 V) and nitrogen was used as a sheath and drying gas at 18.85 and 58.89 L/min and ion gauge was $0.76 \times 10^{e-5}$ Torr. The spectrometer was calibrated in the positive ion mode. Mass spectra of LYC and its oxidised products were acquired with an *m/z* 0–700 scan range. Due to unavailability of reference standards for LYC oxidative products and constraint in the sample size, the possible structure of COL products were predicted and characterized using Chemsketch 8.0 software (ACD Labs, USA).

2.7. Cell biological assays used to assess toxicity and apoptosis inducing activity

2.7.1. Cell lines and culture conditions

Cancer cell lines (PC-3, MCF-7, A431, $HepG_2$, HeLa and A549) were purchased from National Centre for Cell Science, Pune, India. Cells were grown in DMEM, MEM or Ham's F-12 media, containing 10% FBS (GIBCO BRL, USA), 4 mM L-glutamine, and antibiotics (40 μg/mL penicillin and 40 U/mL streptomycin) at 37 °C in a humidified atmosphere of 5% CO_2 in the air.

2.7.2. Cell viability assay

Exponentially growing cells (70–80%) were seeded (5×10^3 cells/well) in the 96-well plate containing 200 μL of culture medium. After 24 h of incubation, media was removed and replenished with 200 μL of the media containing purified LYC (1–50 μM) dissolved in THF (0.5%) and incubated for 48 h (Kotake-Nara et al., 2001). Based on the effect of LYC, three cell lines (PC-3, MCF-7 and HeLa) with maximum reduction in cell viability were chosen for further experimentation. Cells treated separately either with LYC (50 μM), or its equivalent relative concentration of AOL or COL or without carotenoid (control) were incubated for 24 h. The reason for selection of higher concentration of LYC for cell treatment was, LYC may lead to generation of cleavage products at a lower concentration with lesser stability. These compounds are reported to decompose quickly than the major fragments (Lakshminarayana et al., 2013). The final concentration of THF in the culture medium was 0.5%, and control culture received a same concentration of THF alone. Then, the influence of LYC oxidation products on cell viability was evaluated as described above. Further, to support the effect of LYC, AOL and COL on cell death was determined by trypan blue dye exclusion assay (Palozza et al., 2002). In brief, cells were cultured (1×10^5 cells/mL) and treated with increasing concentrations (1, 5, 10, 25 and 50 μM) of LYC, AOL and COL and incubated for 24 h. Then cells were trypsinized, collected and the number of viable cells and dead cells were determined by trypan blue staining (0.4%) using haemocytometer. Experiments were repeated three times independently (data not shown).

2.7.3. Measurement of glutathione and malondialdehyde (MDA) levels in cells

A standard solution of glutathione, as well as cell lysates from control and cells treated with LYC, AOL or COL were incubated with the solution containing NADPH (12 mM), 0.1 mM DTNB and 50 U/L

glutathione reductase (GR). The working solution was prepared in 100 mM sodium-phosphate buffer with 5 mM sodium EDTA (pH 7.4). Oxidised glutathione is reduced by NADPH in the presence of GR and sequentially oxidised by DTNB. The rate of 5-thio-2-nitrobenzoic acid formation was measured at 412 nm and glutathione levels (nmoles/mg protein) present in the sample were recorded by using a standard curve (Tietze, 1969). For lipid peroxides estimation (nmoles MDA/mg protein), cells were trypsinized and the pellets were lysed using tris-buffer (50 mM), EDTA (15 mM) with the protease inhibitor. The cell lysate was used to measure MDA (Ohkawa et al., 1979). Protein was estimated according to Lowry's method (Lowry et al., 1951).

2.7.4. Cell cycle analysis

Cell cycle analysis was done by flow cytometry Sowmya et al. (2015). In brief, control and treated cells (1×10^5 cells/well in 12 well plate) after 24 h incubation were rinsed with PBS and detached with trypsin- EDTA at room temperature and centrifuged at 2500 rpm for 5 min. The cells were washed twice with PBS and resuspended in 1 mL ice-cold hypotonic solution, containing 0.1% triton X-100, 0.1% citrate buffer and 0.1 mg/mL RNase and 50 μg/mL propidium iodide and incubated for 15 min at 37 °C in the dark. After incubation, the cell suspension was analysed for the relative proportions of cells with DNA content diploid G0–G1 (2n), S phase (>2n but <4n), G2/M phase (4n) and sub-G1 peak (sub-diploid cells). The cell cycle distribution were measured with FACS verse flow cytometer (BD Biosciences, San Jose, CA, USA) and analysed by FACS Diva Analysis software.

2.7.5. Apoptosis detection

Apoptosis detection was made with an FITC Annexin-V apoptosis detection kit according to the manufacturer's instructions. Briefly, control and treated cells (1×10^5 cells/well in 12 well plate) after 24 h of incubation were collected, washed with ice-cold PBS and centrifuged at 2500 rpm for 5 min. The cell pellet was resuspended in the ice-cold 1X binding buffer and incubated with FITC-conjugated Annexin V and Propidium iodide (PI) for 15 min at room temperature in the dark. Prior to the analysis, the instrument was calibrated with internal controls, such as unstained cells (negative control) and stained cells (annexin-FITC, PI and annexin-FITC with PI control cells), respectively. The samples were immediately analysed on FACS verse flow cytometer using the Diva analysis software.

2.7.6. ROS detection by flow cytometry

Control and treated cells (2×10^5) were harvested after 24 h of incubation, washed with PBS, and suspended in 1 mL PBS. Followed by DCFH-DA (10 μM) was added and incubated for 15 min in 5% CO_2 at 37 °C. After incubation, cells were washed and resuspended in PBS and were analysed within 1 h on FACS Verse Flow Cytometer. Each cell type was analysed by flow cytometry after calibration with negative (unstained) and positive control (H_2O_2) cells. The results were expressed as fluorescence intensity of dichlorofluorescein compared with control, treatment and cells with H_2O_2 (positive control).

2.7.7. Fluorescence morphological examination by AO/EB and DAPI

The cellular morphological changes were studied by using fluorescence microscope (CKX 41, Olympus Inverted Trinocular Fluorescence Microscope, Japan). Cells were seeded (50,000 cells/100 μl) onto a poly-D lysine coated cover slips and cultured to about 75% confluency. Cells with and without COL was incubated for 24 h at 37 °C and 5% CO_2, dual stained with 1:1 ratio of acridine orange (100 μg/mL) and ethidium bromide (100 μg/mL) for 5 min. For further confirmation, nuclei staining were done with 1 μg/mL DAPI for 3 min in the dark. The cells were then washed with PBS three times. The morphology of cell nuclei was observed and images were documented using Q-Imaging MP3.3 cooled colour camera with Q-Capture Pro7 imaging software (Canada).

2.8. Statistical analysis

Data were tested for homogeneity of variances by the Bartlett test. When homogenous variances were confirmed, the data were tested by ANOVA and significant differences between the groups were evaluated by Tukey's test. The difference in mean was considered significant at $p < 0.05$.

3. Results

3.1. Isolation and characterization of LYC, and its possible oxidation products

LYC isolated from ripened tomato puree was compared with analytical standard and confirmed its characteristic UV-spectra, λ_{max}, retention time and mass spectra. The LYC content in the tomato puree was 9.01 ± 0.65 mg/100 g wet weight and the purity of isolated LYC was found to be 93 ± 5% as per the UPLC analysis as indicated in Fig. 1. The autoxidation of LYC resulted in isomerization and there was no degradation has observed previously (Arathi et al., 2015a). Further, LYC (5 mg/5 mL) was oxidised with $KMnO_4$ incubation, and the optical density of the sample was measured. The results showed that after incubation, LYC concentration depleted with increased duration of exposure time, indicating degradation of LYC, follows first-order degradation whereas, LYC has followed a zero-order degradation kinetics (Fig. 2). An initial (0-min exposure) LYC concentration (5 mg/mL) was measured at 470 nm which decreased to less than 0.5 mg/5 mL after 40 min exposure, indicating that LYC was involved in a chemical reaction with $KMnO_4$ or radicals generated from it (Fig. 2a), which resulted in a change in its chemical structure (spectral change) (Fig. 2b). The LYC was readily oxidised to form the mixture of oxidative products, the degradation was due to break in its conjugated double bond in the polyene chain. The oxidised or break down products can be formed either primary or secondary reactions. The characteristic UV–visible spectra lend further support to the hypothesis that LYC is degraded to various oxidative products either with single or multiple step reactions (Figs. 2 and 3). The possible characteristic fragmented ions, formed from LYC in the samples exposed to $KMnO_4$ are shown in Table 2. The residual LYC after 16 min of incubation with $KMnO_4$ was 50% remaining 50% was oxidised under described conditions, were further used for cell culture studies. The MS profile of LYC oxidation products are shown in Fig. 3. The MS analysis of negative control (without LYC) showed no cross contamination of $KMnO_4$ and showed only base line peak.

3.2. Effect of lycopene on cell viability

The effect of LYC on cell viability of different cancer cell lines was shown in Fig. 4. LYC (1–50 μM) inhibited the cell growth dose-dependently (up to 5 μM) when compared to respective controls. The cell viability of all the cancer cell lines treated with 5 μM LYC was significantly different ($p < 0.05$) when incubated for 48 h. It was observed that there is no significant difference in cell number between untreated and vehicle control. Result suggest that THF (0.5%) stabilized with BHT did not affect the cell growth. However, upon LYC treatment, the percent cell viability in different cancer cell lines was observed in the order of PC-3 (47.3%) < HeLa (33.7%) < MCF-7 (29.8%) < A549 (27.3%) < $HepG_2$ (22.9%) < A431 (17.8%). Among cancer cell lines, a significant reduction in cell

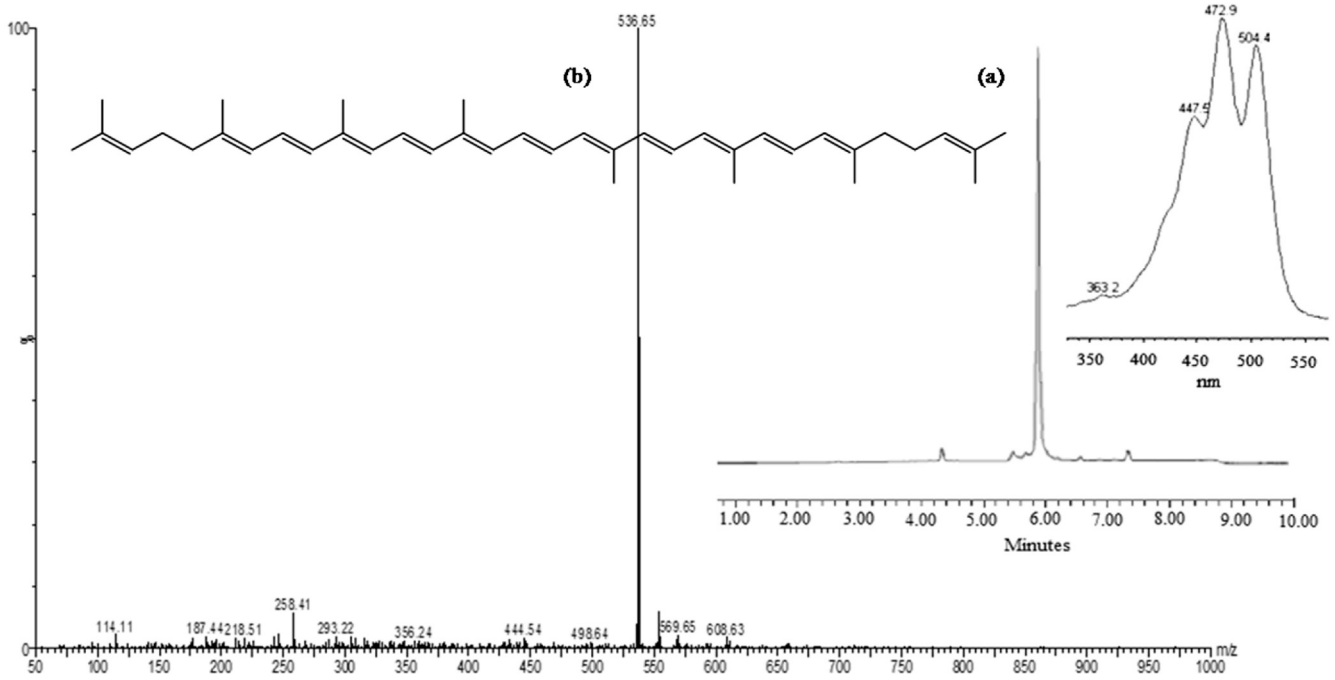


Fig. 1. A typical UPLC profile and UV spectra (a) MS analysis of purified lycopene (b).

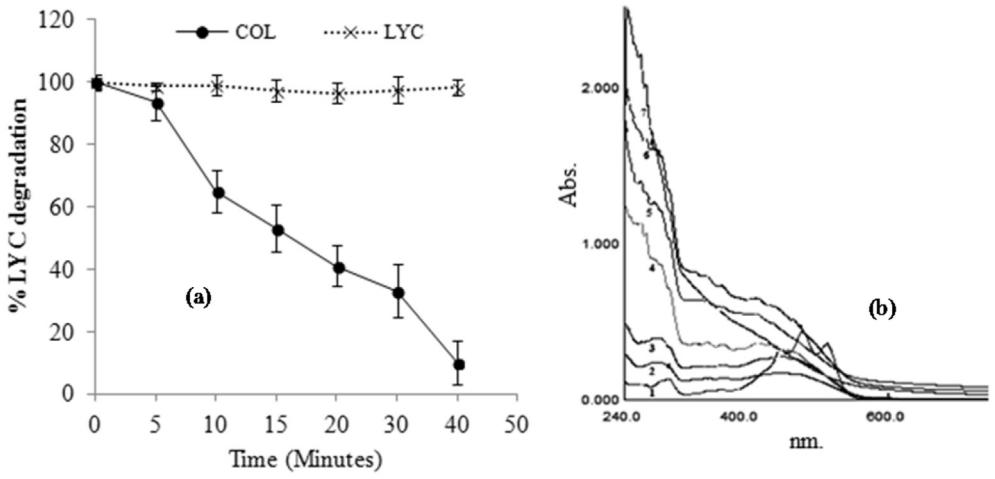


Fig. 2. $KMnO_4$ induced degradation pattern of lycopene at different time points (a) and their overlaid UV absorbance spectra (b). Values are mean ± SD of three samples.

viability was found in PC-3, HeLa and MCF-7 cells. Among cell lines tested cell cytotoxicity of LYC was lower in $HepG_2$ and A431 cells. These data demonstrate that the effect of LYC on cell viability was mostly dependent on cell type and dosage. Hence, these cell lines were treated further to evaluate the influence of LYC oxidative products on ROS generation and induction of apoptosis.

3.3. Effect of LYC oxidative products on cell viability

The influence of LYC and LYC oxidative products (AOL or COL) on cytotoxicity of PC-3, HeLa and MCF-7 cells were shown in Fig. 5. The COL strongly inhibited the cell viability compared to AOL and LYC. The % cell viability of PC-3 cells were decreased by 29.6 (LYC), 39.2 (AOL) and 71.4% (COL) compared to control. Likewise, 29.3, 44.2 & 68.4% decrease in MCF-7 and 32.6, 29.4 & 56.2% decrease in HeLa cells was evident compared to control respectively (Fig. 5). In addition to this, cell death was measured by tryphan blue dye exclusion that strongly confirmed the above results (data not shown). The IC_{50} concentration of COL was found to be 35.0, 36.5, 44.4 μM respectively in PC-3, MCF-7 and HeLa cells.

3.4. Oxidation status of LYC, AOL and COL treated cells

Results of glutathione levels in PC-3 cell lines was lower by 15.5 (LYC), 9.7 (AOL) and 49.5% (COL) respectively than control (Fig. 6). It is evident that the glutathione levels in, LYC oxidative products treated culture decreased significantly by 34 and 39.8% in COL than LYC and AOL indicating that COL is superior to LYC and AOL. The effect of LYC and LYC oxidised products on GSH levels in MCF-7 cells were shown in Fig. 6. Glutathione levels of control cells were higher by 30.4, 36.0 and 50.4% than LYC, AOL and COL, respectively. Similarly, in the case of HeLa cells, the levels of GSH in control cells

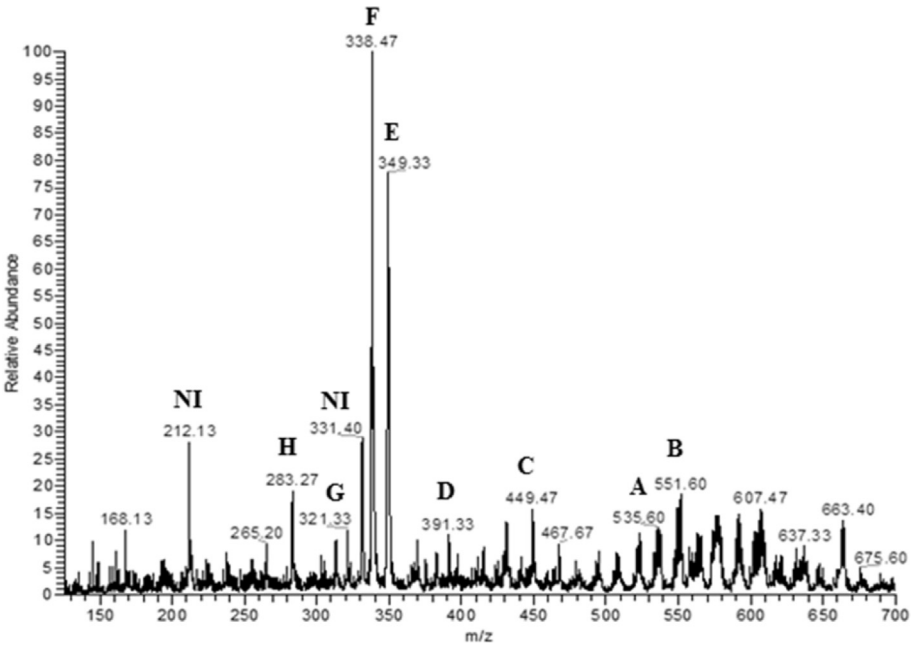


Fig. 3. APCI^{+ve} - Mass Spectra profile of $KMnO_4$ induced LYC oxidised products. Refer Table.1 for respective molecular mass and possible structures of A-H (NI-Not identified).

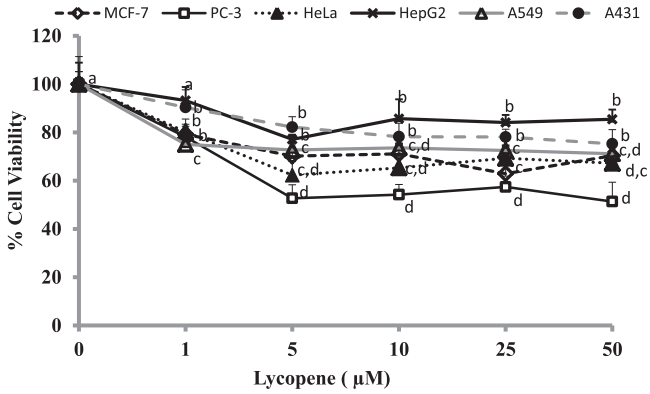


Fig. 4. Effect of lycopene (1–50 µM) on viability of different cancer cell lines. Cells were treated with various concentrations of lycopene for 48 h ^{a,b,c,d} Values not sharing common letters are significantly different ($p < 0.05$) between cell lines as determined by two-way ANOVA followed by Tukey's test. Values are mean ± SD of five samples.

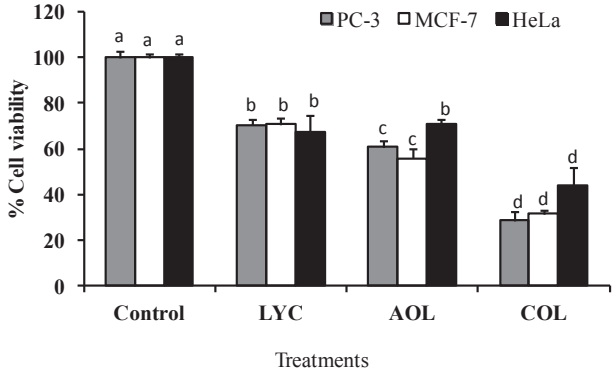


Fig. 5. Effect of LYC, AOL and COL on the viability of MCF-7, PC-3 and HeLa cell lines treated for 24 h ^{a,b,c,d} Values not sharing common letters are significantly different ($p < 0.05$) between treated and control samples as determined by two-way ANOVA followed by Tukey's test. Values are mean ± SD of five samples.

were higher by 14, 22.2, and 59.2% than LYC, AOL and COL, respectively.

Correspondingly, the influence of LYC oxidised products on lipid peroxides (MDA) level in treated cells was shown in Fig. 6. The MDA

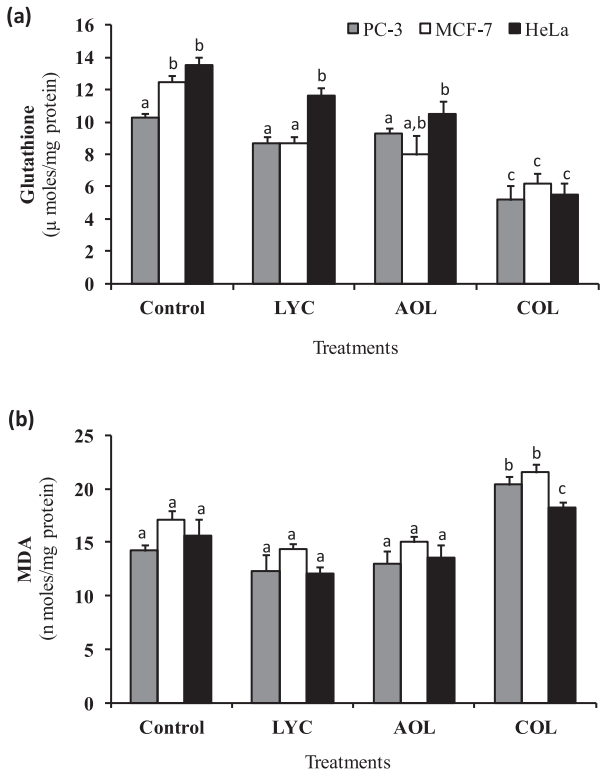


Fig. 6. Influence of LYC, AOL and COL on glutathione (a) and MDA (b) levels in MCF-7, PC-3 and HeLa cells treated for 24 h ^{a,b,c} Values not sharing common letters are significantly different ($p < 0.05$) between treated and control samples as determined by two-way ANOVA followed by Tukey's test. Values are mean ± SD of five samples.

Table 1
Influence of LYC and COL on cell cycle progression and apoptosis in MCF-7, PC-3 and HeLa cell lines.

Experimental groups	Cell cycle distribution (%)				Apoptosis (%) (EA + LA)*
	Sub G_1	G_0/G_1	S	G_2/M	
MCF-7 cells					
Control	2.1 ± 1.2[a]	72.6 ± 1.5[a]	11.6 ± 1.2[a]	13.8 ± 1.9[a]	9.9 ± 1.9[a]
LYC	1.9 ± 1.8[a]	73.7 ± 0.5[a]	10.5 ± 0.4[a]	13.5 ± 1.6[a]	12.2 ± 1.3[a]
COL	18.5 ± 2.1[b]	53.5 ± 0.8[b]	13.5 ± 1.4[b]	15.0 ± 1.4[a]	31.1 ± 1.7[b]
PC-3 cells					
Control	0.9 ± 0.4[a]	71.3 ± 0.5[a]	12.6 ± 0.7[a]	14.6 ± 1.8[a]	5.1 ± 1.3[a]
LYC	1.2 ± 1.2[a]	72.4 ± 0.8[a]	12.5 ± 0.5[a]	13.5 ± 1.6[a]	8.1 ± 1.8[b]
COL	15.5 ± 1.5[b]	57.9 ± 0.4[b]	12.6 ± 0.7[a]	14.0 ± 0.7[a]	22.0 ± 1.5[c]
HeLa cells					
Control	1.2 ± 1.8[a]	72.0 ± 1.3[a]	12.1 ± 0.7[a]	14.2 ± 1.5[a]	10.1 ± 1.3[a]
LYC	1.8 ± 1.2[a]	73.3 ± 0.8[a]	12.8 ± 1.2[a]	12.1 ± 1.4[a]	9.7 ± 1.9[a]
COL	12.5 ± 1.2[b]	59.8 ± 2.5[b]	13.6 ± 0.9[b]	14.1 ± 0.9[a]	21.8 ± 1.5[b]

The values are mean ± SD ($n = 3$).
*EA-Early apoptosis, LA-Late apoptosis.
[a,b,c] Values not sharing a common superscript letters within a column under each type of cells treatment are significantly different ($p < 0.05$) from their respective control samples, as determined by one-way ANOVA followed by Tukey's test.

levels in PC-3 cells treated with COL were significantly higher by 30.3, 39.7 and 36.2% than control, LYC and AOL. Likewise, MDA levels in MCF-7 cells treated with COL was higher by 20.4, 33.0 and 30.2% than control, LYC and AOL. In the case of HeLa cells, COL treated cells shown higher levels of MDA formation by 14.2, 33.5 and 25.8% than control, LYC and AOL, respectively (Fig. 6).

3.5. Apoptosis induction of lycopene oxidation products

Based on the results of cytotoxicity and oxidative status, it was observed that there is no significant difference between AOL and LYC. Hence, hereafter COL was used for further experimentation to evaluate its efficiency against anti-proliferation of cancer cell lines. Cell cycle analysis was carried out in control and cells treated with LYC (50 μM) or its relative concentration of COL for 24 h. An increase in apoptosis was observed in PC-3, MCF-7 and HeLa cells treated with COL (Table 1). Further, FACS analysis demonstrated that COL treated cells showed an increase in Sub-*G1* peak representing the apoptotic cell population. The influence of COL on apoptosis was confirmed further by Annexin FITC-PI staining and shown in Fig. 7 and Table 1. Results of fluorescence microscopic observation showed an early and late apoptosis, morphological changes and nuclear condensations in COL treated cells (Fig. 9). The live cells were stained uniformly green, early apoptotic cells were green with

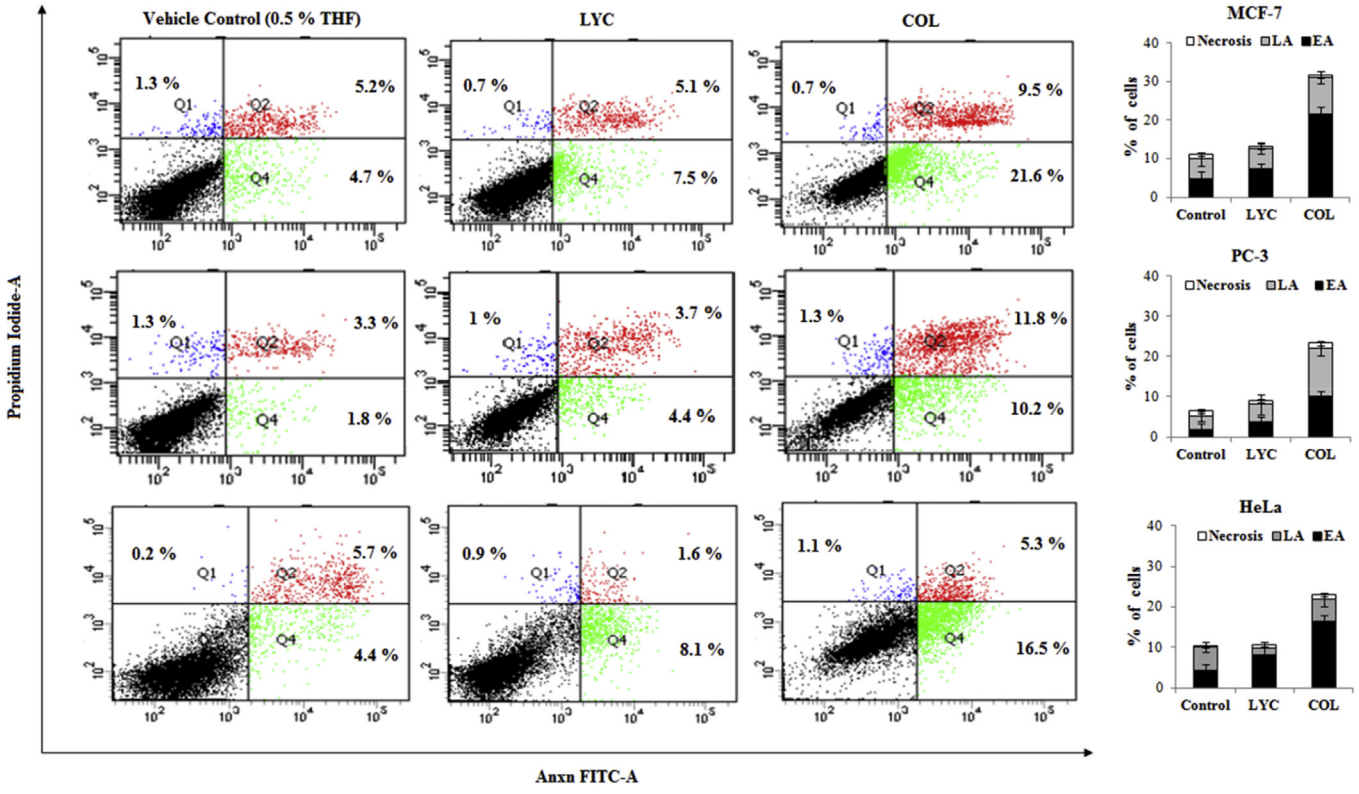


Fig. 7. Detection of apoptosis in MCF-7, PC-3 and HeLa cells treated with LYC and COL products. Cells were stained with annexin V-FITC and PI, and analysed by flow cytometry. In each panel, Q4 quadrant shows annexin V positive cells which are in early stage of apoptosis, Q2 shows both annexin V and PI positive, which are in the late stage of apoptosis, Q1 shows PI positive cells which are dead. Bar diagram represents the distribution of early, late and necrotic cell populations. Values are mean ± SD of three samples.

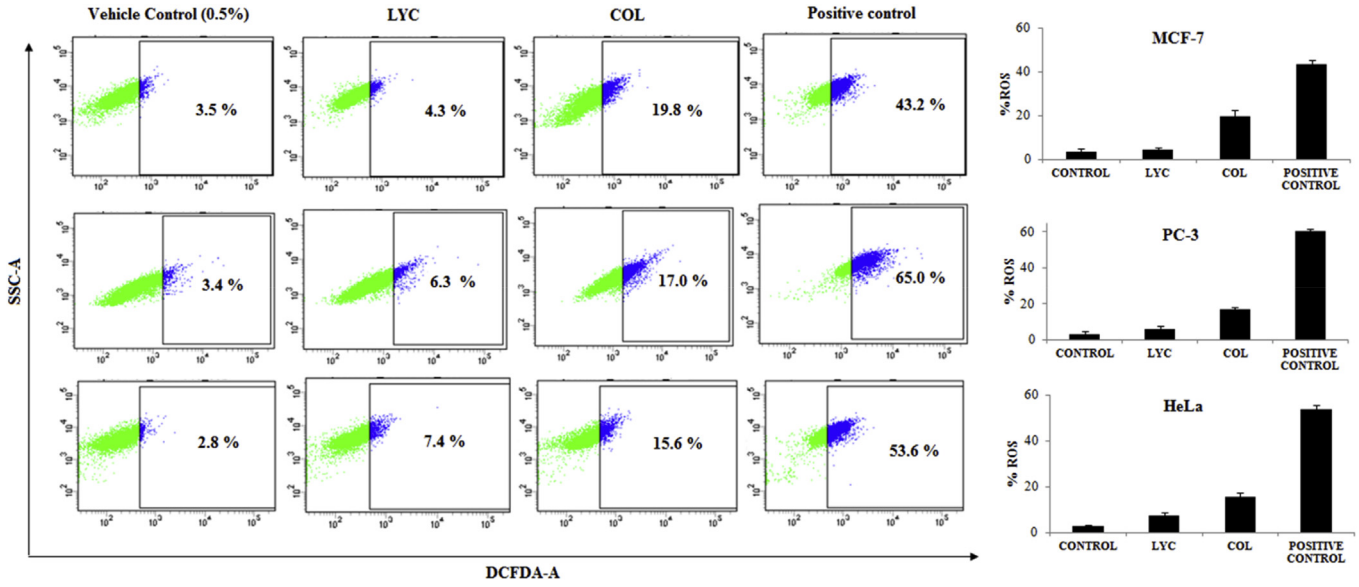


Fig. 8. Flow cytometry analysis showing levels of intracellular ROS generation in MCF-7, PC-3 and HeLa cells treated with LYC and COL using DCFH-DA assay. Values are mean ± SD of three samples.

bright green nuclei due to chromatin condensation and nuclear fragmentation. Late apoptotic cells were stained orange with nuclear condensation.

3.6. Intracellular ROS generation

Influence of LYC and its oxidation products (COL) on the levels of intracellular ROS in three different cell lines were shown in Fig. 8. In all the cases, COL increased the ROS levels than the LYC and control treated cells. In the case of MCF-7 cells, the levels of ROS in control and LYC were lower by 16.3 and 15.5% than COL treated cells. This may be the reason for higher cell death in COL treated cells. Similarly, in PC-3 cells, the ROS levels were lowered by 13.6% in control and 10.7% in LYC compared to COL. In case of HeLa cells, ROS levels were lower by 12.8% in control and 8.2% in LYC than the COL treated cells respectively. The % of ROS in positive control was found to be 43.2, 65.0 and 53.6% in MCF-7, PC-3 and HeLa cells, respectively.

4. Discussion

Possible biological functions of carotenoid oxidative products or metabolites are currently an active area of investigation in nutrition and biomedical research. Recent advancement in carotenoid metabolism necessitated to hypothesize that, carotenoid metabolites/oxidative products are responsible for bioactivity (Arathi et al., 2015b). Several carotenoid metabolites including LYC have been identified in vitro and in vivo (Khachik et al., 1997; Kim et al., 2001; Caris-Veyrat et al., 2003; Hu et al., 2006; Kopec et al., 2010; Nidhi et al., 2015). In this study, we identified and elucidated possible LYC oxidative products induced by $KMnO_4$. The fragmented ion lycopene 1, 2 epoxide (designated as compound B) detected in this study may also be formed in vivo due to metabolic oxidation of LYC (Rodriguez and Rodriguez-Amaya, 2009). The similar compound was also formed by oxidation when LYC incubated with *m*-chloroperbenzoic acid (Rodriguez and Rodriguez-Amaya, 2009). Previously, Caris-Veyrat et al. (2003) have characterized LYC oxidation products in vitro by $KMnO_4$ and atmospheric oxygen catalyzed by metalloporphyrin. They have postulated the formation of LYC metabolites (apo-lycopenal, apo-lycopenone and apo-carotendials) in vivo. Further, ionization and mass of respected fragmented ions can vary under optimization of mass spectrometer conditions (Sowmya et al., 2014). Correspondingly, Pennathur et al. (2010) identified HOCl-mediated oxidative products of LYC (apo-lycopenal, apo-lycopenic acid and apo-carotendials) and proposed the cleavage pattern. We also found few similar LYC oxidation products (C, D, E, and H) as they reported earlier (Table 2). Compound F and G were found to be new molecules and designated as (4E, 6E, 8E, 10E, 12E, 14E)-2, 7, 11, 15, 19-pentam ethylicosa-2, 4, 6, 8, 10, 12, 14, 18-octaene and (2E, 4E, 6E, 8E, 10E,12E,14E,16E)-2, 6, 11,15-tetramethyloctadeca-2, 4, 6, 8,10,12, 14, 16-octaenedial. The detailed characterization of these compounds could not be carried out due to quantitative limitations and stability issues. Since not many studies are available on the biofunctionality of oxidation products of LYC and being similar kind of molecules reported in the biological system (Kopec et al., 2010). We limited our studies with MS analysis and composite oxidised LYC products for evaluation of their functions.

The cytotoxic studies demonstrated that LYC is comparatively effective at > 5 μM in various cancer cell lines studies (Fig. 4). The varied percent cytotoxicity of LYC in different cancer cell lines may be due to the difference in cell growth modality, cell metabolic status, membrane composition and level of carotenoid accumulation (Sowmya et al., 2015). Earlier, Teodoro et al. (2012) shown a significant decrease in number of viable cells after LYC treatment for 48 h inHT-29, T84 cells and MCF-7 cell lines among eight different cancer cell lines (HT-29, T84, MCF-7, DU145, A549, HepG2, HeLa and Hep2). In addition, they also observed discreet effect of LYC at 5 μM in DU 145 and HeLa cells, and no effect was found in the other cells. Furthermore, they concluded effect of lycopene on anti-proliferation of cancer cells may be due to cellular type, time and dose.

Likewise, in the present study we observed significantly decreased cell viability in MCF-7, PC-3, and HeLa cell lines. The variable results of cell viability compared to others report Teodoro et al. (2012) may be attributed due to lycopene source or mode of delivery used for the experiment. The source of LYC used by Teodoro et al. was commercially obtained whereas in this study it was purified from tomato source. In continuation, Soares et al. (2014) evaluated role of lycopene on induction of apoptosis in human prostate cancer (PCa) cells and observed the up-regulation of Bax

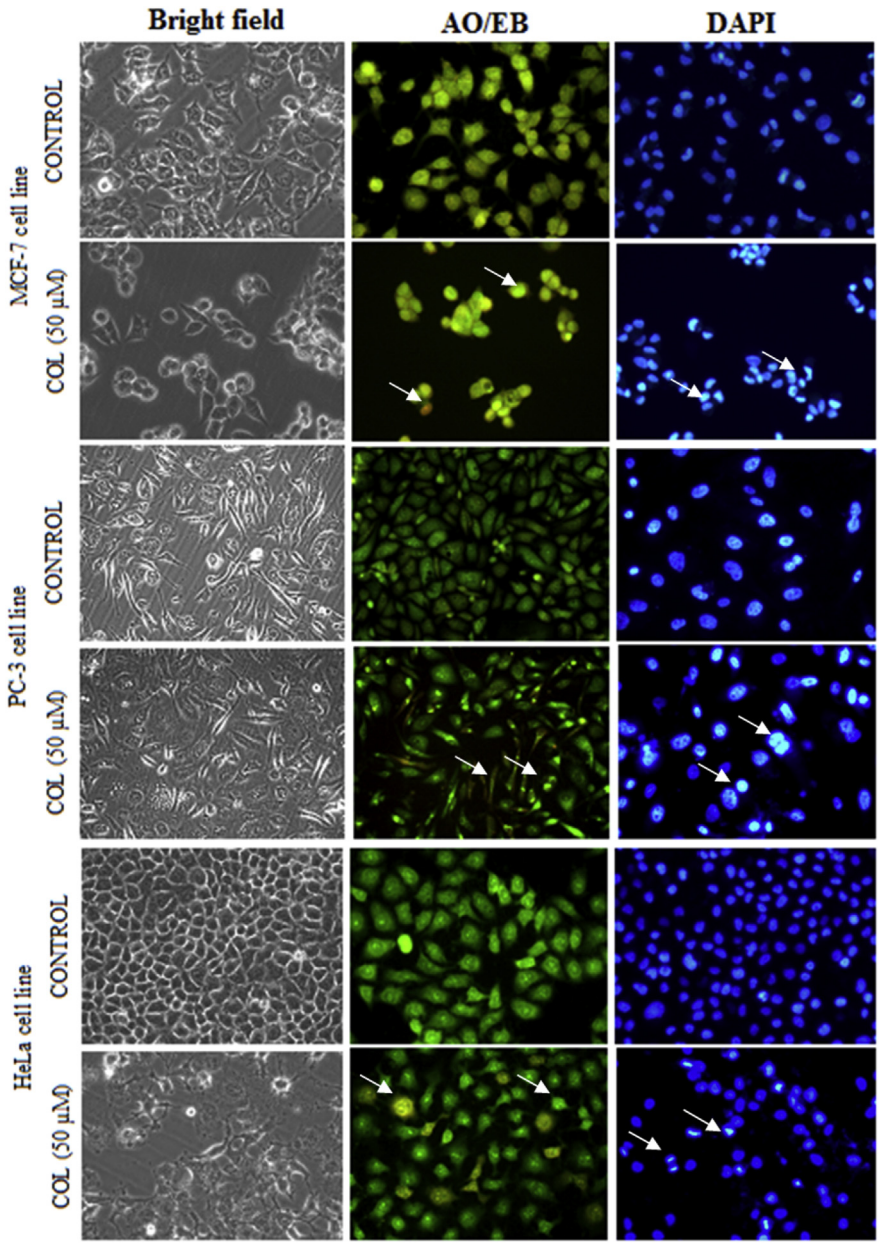
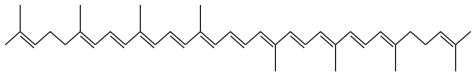
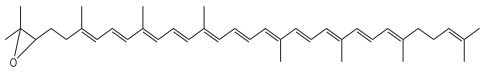
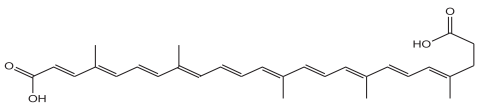
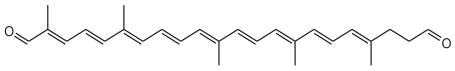
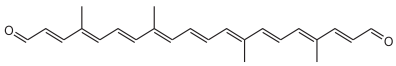
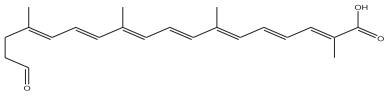
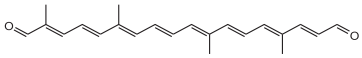
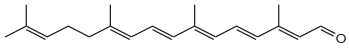


Fig. 9. Morphological observation of COL treated MCF-7, PC-3 and HeLa cells with acridine orange/ethidium bromide staining, (200x) arrows indicate early and late apoptotic cells. DAPI was used as a nuclear marker. Note: The microscopic field for AO/EB and DAPI are different with the same cell lineages.

and down regulation of Bcl-2 gene expression in PCa cells. Studies have correlated the effect of LYC in the reduction of prostate cancer (Kim et al., 2002; Etminan et al., 2004; Gunasekera et al., 2007; Ivanov et al., 2007). The results obtained may be partly due to the metabolites/oxidation products of LYC. However, studies related to role of metabolites/oxidation products on molecular mechanism such as cyclins and the inhibitor of apoptosis proteins (IAPs), needs to be addressed. In contrast, few reports have shown the limited effect of LYC in prostate and other cell lines (Forbes et al., 2003; Burgess et al., 2008). These studies show that effect of LYC was found to be highly variable may be due to variations of in vitro techniques, cell types, culture conditions, stability and solubility of LYC in cell culture systems that make it very difficult to understand the mechanism of action (Ford et al., 2011). As observed in previous study (Lakshminarayana et al., 2010), here we have noticed concentration of LYC studied and LYC oxidation products did not affect the normal cells under the experimental conditions. Further, the effect of LYC oxidation products on anti-proliferation of different cancer cell lines are less explored. Although the higher concentration of LYC has not shown a detrimental effect on cell viability than 5 μM in all the three cell lines, the higher concentration of LYC was used for cell treatment, since oxidation of carotenoid may lead to the generation of smaller fragments with lower concentration and lesser stability (Lakshminarayana et al., 2013). We identified and interpreted for the first time that COL products exhibited higher anticancer property on MCF-7, PC-3 and HeLa cell lines. The cytotoxic effect of COL was found to be more pronounced which may be due to change in its polarity than the intact LYC. It was observed that LYC and AOL had minimal effect since they are hydrophobic in nature and forms aggregation. Earlier, Hu et al. (1998) identified 5, 8-Endoperoxy-2, 3-dihydro-β-apocarotene-13-one an oxidation product of β-carotene and shown its growth inhibition of breast

Table 2
Fragmented ions derived from LYC after incubation with $KMnO_4$, elucidated by MS APCI $^{+ve}$ mode showing their possible molecular structure, molecular mass and molecular formula.

Alphabet	*m/z*	Proposed structure	Formula	App. Mol. Wt.	Name
A	537.44		$C_{40}H_{56}$	536.43	Lycopene
B	553.4		$C_{40}H_{56}O$	552.4	2,2-dimethyl-3-(3E, 5E, 7E, 9E, 11E, 13E, 15E, 17E, 19E, 21E, 23E) -3,7, 11, 16, 20, 24, 28 – heptamethylnonacosa -3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 27-dodecaenyl) oxirane
C	449.2		$C_{29}H_{36}O_4$	448.59	(2E, 4E, 6E, 8E,10E, 12E, 14E, 16E, 18E, 20E)-4, 8, 13, 17, 21 – pentamethyltetracosa -2, 4, 6, 8, 10, 12, 14, 16, 18, 20-decaenedioic acid
D	391.2		$C_{27}H_{34}O_2$	390.2	Apo-1,6′-carotendial
E	349.2		$C_{24}H_{28}O_2$	348.2	Apo-5,6′-carotendial
F	340.4		$C_{22}H_{28}O_3$	339.2	(2E, 4E, 6E, 8E, 10E, 12E, 14E)-17-formyl-2, 7, 11, 15-tetramethylheptadeca-2, 4, 6, 8, 10, 12, 14-heptaenoic acid
G	323.1		$C_{22}H_{26}O_2$	322.1	(2E, 4E, 6E, 8E, 10E,12E,14E,16E)-2, 6, 11, 15-tetram ethyloctadeca-2, 4, 6, 8, 10, 12, 14, 16-octaenedial
H	285.2		$C_{20}H_{28}O$	284.2	(2E, 4E, 6E, 8E, 10E) - 3, 7, 11, 15 -tetramethylhexadeca - 2, 4, 6, 8, 10, 14 -hexanal

cancer cells. Likewise, 5, 6-epoxy-β-carotene was reported to have a greater differentiation inducing activity than β-carotene in leukaemia cells (Duitsman et al., 1999). Hanusch et al. (1995) have reported an oxidation product (4-oxo-retinoic acid) of canthaxanthin activates retinoic acid receptor-β gene and enhance gap junctional communication in murine fibroblast cells. In the current study, we observed the accumulation of cells at sub-*G1* peak in COL treated cell lines during cell cycle regulation. Further, cell death by apoptosis was shown with an increase in both early and late apoptotic populations compared to control and LYC (Table 1 and Fig. 7). Previously, Nara et al. (2001) have showed the involvement of acyclic carotenoid oxidation products on cancer cell growth inhibition. Likewise, Zhang et al. (2003) demonstrated the role of LYC cleavage product, (*E, E, E*)-4 methyl-8-oxo-2, 4, 6-nonatrienal on induction of apoptosis through down regulation of Bcl-2 and Bcl-XL expression in HL-60 cells. While, Aust et al. (2003) shown the implication of LYC oxidation product (2, 7, 11-trimethyl-tetradeca-hexaene-1, 14 dial) on cell signalling via gap junctions in WB-F344 cells. Here, COL treated MCF-7, PC-3, HeLa cells showed increased apoptosis with 3.1, 4.3, 2.1 fold than control, likewise 2.4,

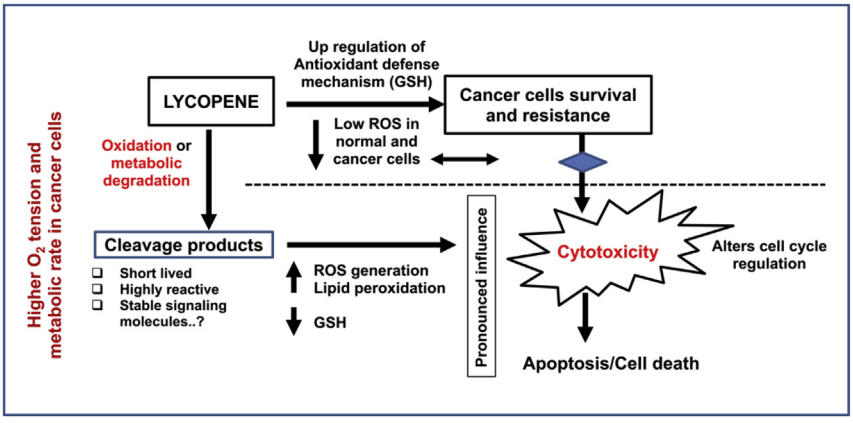


Fig. 10. Scheme of redox status and proposed therapeutic approach on cytotoxic effect of lycopene cleavage products by modulation of ROS and programmed cell death in cancer cells.

2.7, 2.2 folds increased apoptosis was observed than LYC, respectively. The higher percent of apoptosis found in this study was presumed due to an increased oxidative status of cancer cells as shown by higher cellular levels of MDA and depleted glutathione in COL treated cells than control and LYC (Fig. 6). The depletion of glutathione and increases in MDA levels, reflects lipid peroxidation within mitochondria, indicating an increase in oxidative stress (Siems et al., 2002). Further, the elevated accumulation of reactive oxygen species induces oxidative damage to proteins, including the adenine nucleotide translocator, lipids, and DNA molecules in subcellular organelle like mitochondria, and nucleus (Siems et al., 2002). Carotenoids are known to act as radical scavengers and have been noted relevant use to modulate altered physiology or biochemical changes in complications like cancer. In contrast, carotenoids are shown to behave as pro-oxidant under certain situations in vitro and in vivo. The antioxidant/pro-oxidant properties of carotenoids are complex and can vary with the chemical environment and nature of carotenoid species (El-Agamey et al., 2004). Possibly, peroxyl radicals (ROO•) can covalently add to the carotenoid molecule forming an adduct (ROOCAR•) or peroxyl radical can abstract a hydrogen atom from the carotenoid to form a carotenoid radical (CAR•) (Prasad et al., 2006). Further, carotenoid also transfers an electron to the peroxyl radical, so that the carotenoid becomes a radical cation ($CAR^{\bullet+}$) and these radicals can react further in various ways. Previously, we revealed the presence of shorter polyene chain cleavage products from lutein derived from either photo or UV oxidation react with peroxy radicals through a number of reaction mechanisms (Lakshminarayana et al., 2008, 2013; Nidhi et al., 2015). In the present study, the induction of apoptosis by COL may positively correlate with intermediate production of intracellular ROS than lycopene treated and vehicle control cells (Fig. 8). Further, in agreement with previous report Prasad et al. (2006), we hypothesized that ROS generation and levels may profoundly alter the pro-apoptotic proteins and induce apoptosis (data not shown). Studies also have shown that β-carotene at higher concentration acts as pro-oxidants and induce apoptosis by increasing intracellular ROS levels (Palozza et al., 2001; Cui et al., 2007). Previously, Siems et al. (2002) reported that β-carotene cleavage products are involved in carcinogenic effect by impairing mitochondrial functions under high oxidation stress in rat and also proposed the mechanism of pro-oxidant effect of β-carotene. In addition, microscopic observations of COL treated cell lines in this study confirmed the apoptosis by typical morphological changes in cells and DNA condensation (Fig. 9). These results suggest that oxidation products of LYC may have a significant role against cancer cells proliferation. As per our knowledge and available literature there is no information related to the concentration of carotenoids oxidation products on biological functions. Based on current observation, we proposed the therapeutic approach of LYC oxidation products and their possible mechanism on progression of cytotoxicity and cell death in cancer cells (Fig. 10). Further, isolation, separation and characterization of individual oxidation product is required to determine its chemical and biological properties (under standardization).

To conclude, there are several unknown metabolites or oxidation products of LYC, apart from the major ones, that may be involved in the reduction of cell proliferation, through modulating cell cycle progression. In this study, we have demonstrated that the COL products are potent in the induction of apoptosis with intermediate ROS generation than intact LYC and AOL. The effect of COL could be synergistic or due to the presence of individual bioactive fragmented compounds of LYC. These results suggested that oxidation products of carotenoids may attribute potential health benefits. Further, detailed characterization and stability of these oxidation products in a biological environment is challenging and deserves future studies to understand specific biological function.

Acknowledgments

Mrs Arathi B.P. acknowledges Department of Science and Technology, Govt. of India. for the grant of Women Scientist Fellowship (F.NO.SR/WOS-A/LS-35/2012). Authors acknowledge the Department of Biotechnology-Bangalore University, UGC-SAP and DST-FIST facilities. Authors also acknowledge Indian Institute of Science for FACS facilities, and Professor T. Ramasharma and Dr. D. Lakshmanan for critical review of the manuscript with respect to subject and English language.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.fct.2016.09.016

References

Arathi, B.P., Sowmya, P.R., Vijay, K., Baskaran, V., Lakshminarayana, R., 2015a. Metabolomics of carotenoids: the challenges and prospects – a review. Trends Food Sci. Tech. 45, 105–117.
Arathi, B.P., Sowmya, P.R., Vijay, K., Dilshad, P., Saikat, B., Gopal, V., Lakshminarayana, R., 2015b. An improved method of UPLC-PDA-MS/MS analysis of lycopene isomers. Food Anal. Methods 8, 1962–1969.
Aust, O., Ale-Agha, N., Zhang, L., Wollersen, H., Sies, H., Stahl, W., 2003. Lycopene oxidation product enhances gap junctional communication. Food Chem. Toxicol. 41, 1399–1407.
Burgess, L.C., Rice, E., Fischer, T., Seekins, J.R., Burgess, T.P., Sticka, S.J., Klatt, K., 2008. Lycopene has limited effect on cell proliferation in only two of seven human cell lines (both cancerous and noncancerous) in an in vitro system with doses across the physiological range. Toxicol. vitro 22, 1297–1300.
Burton, G.W., Ingold, K.U., 1984. β-Carotene: an unusual type of lipid antioxidant. Science. 224, 569–573.
Caris-Veyrat, C., Schmid, A., Carail, M., Böhm, V., 2003. Cleavage products of lycopene produced by in vitro oxidations: characterization and mechanisms of formation. J. Agric. Food Chem. 51, 7318–7325.
Cui, Y., Lu, Z., Bai, L., Shi, Z., Zhao, W.E., Zhao, B., 2007. β-Carotene induces apoptosis and up-regulates peroxisome proliferator activated receptor gamma expression and reactive oxygen species production in MCF-7 cancer cells. Eur. J. Cancer 43, 2590–2601.
Duitsman, P.K., Barua, A.B., Becker, B., Olson, J.A., 1999. Effects of epoxy carotenoids, β-carotene, and retinoic acid on the differentiation and viability of the leukemia cell line NB4 in vitro. Int. J. Vitam. Nutr. Res. 69, 303–308.
El-Agamey, A., Lowe, G.M., McGarvey, D.J., Mortensen, A., Phillip, D.M., Truscott, T.G., Young, A.J., 2004. Carotenoid radical chemistry and antioxidant/pro-oxidant properties. Arch. Biochem. Biophys. 430, 37–48.
Emenhiser, C., Sander, L.C., Schwartz, S.J., 1995. Capability of a polymeric C30 stationary phase to resolve cis-trans carotenoid isomers in reversed-phase liquid chromatography. J. Chromatogr. A 707, 205–216.
Eroglu, A., Harrison, E.H., 2013. Carotenoid metabolism in mammals, including man: formation, occurrence, and function of apocarotenoids. J. Lipid Res. 54, 1719–1730.
Etminan, M., Takkouche, B., Caamaño-Isorna, F., 2004. The role of tomato products and lycopene in the prevention of prostate cancer: a meta-analysis of observational studies. Cancer Epidemiol. Biomarkers Prev. 13, 340–345.
Ferreira, A.L., Yeumb, K.J., Russell, R.M., Krinsky, N.I., Tang, G., 2004. Enzymatic and oxidative metabolites of lycopene. J. Nutri. Biochem. 15, 493–502.
Forbes, K., Gillette, K., Sehgal, I., 2003. Lycopene increases urokinase receptor and fails to inhibit growth or connexin expression in a metastatically passaged prostate cancer cell line: a brief communication. Exp. Biol. Med. 228, 967–971.
Ford, N.A., Elsen, A.C., Zuniga, K., Lindshield, B.L., Erdman Jr., J.W., 2011. Lycopene and apo-12′-lycopenal reduce cell proliferation and alter cell cycle progression in human prostate cancer cells. Nutr. Cancer 63, 256–263.
Gajic, M., Zaripheh, S., Sun, F., Erdman Jr., J.W., 2006. Apo-8′-lycopenal and apo-12′-lycopenal are metabolic products of lycopene in rat liver. J. Nutr. 136, 1552–1557.
Giovannucci, E., Rimm, E.B., Liu, Y., Stampfer, M.J., Willett, W.C., 2002. A prospective study of tomato products, lycopene, and prostate cancer risk. J. Natl. Cancer Inst. 94, 391–398.
Gunasekera, R.S., Sewgobind, K., Desai, S., Dunn, L., Black, H.S., McKeehan, W.L., Patil, B., 2007. Lycopene and lutein inhibit proliferation in rat prostate carcinoma cells. Nutr. Cancer 58, 171–177.
Halliwell, B., 1999. Establishing the significance and optimal intake of dietary antioxidants: the biomarker concept. Nutr. Rev. 57, 104–113.
Hanusch, M., Stahl, W., Schulz, W.A., Sies, H., 1995. Induction of gap junctional communication by 4-oxoretinoic acid generated from its precursor cantha-xanthin. Arch. Biochem. Biophys. 317, 423–428.

Herzog, A., Siler, U., Spitzer, V., Seifert, N., Denelavas, A., Hunziker, P.B., Hunziker, W., Goralczyk, R., Wertz, K., 2005. Lycopene reduced gene expression of steroid targets and inflammatory markers in normal rat prostate. FASEB J. 19, 272–274.
Hu, X., White, K.M., Jacobsen, N.E., Mangelsdorf, D.J., Canfield, L.M., 1998. Inhibition of growth and cholesterol synthesis in breast cancer cells by oxidation products of β-carotene. J. Nutri. Biochem. 9, 567–574.
Hu, K.Q., Liu, C., Ernst, H., Krinsky, N.I., Russell, R.M., Wang, X.D., 2006. The biochemical characterization of ferret Carotene-9, 10-Monooxygenase catalyzing cleavage of carotenoids in vitro and in vivo. J. Biol. Chem. 281, 19327–19338.
Ivanov, N.I., Cowell, S.P., Brown, P., Rennie, P.S., Guns, E.S., Cox, M.E., 2007. Lycopene differentially induces quiescence and apoptosis in androgen-responsive and -independent prostate cancer cell lines. Clin. Nutr. 26, 252–263.
Khachik, F., Spangler, C.J., Cecil Smith, J., 1997. Identification, quantification, and relative concentrations of carotenoids and their metabolites in human milk and serum. Anal. Chem. 69, 1873–1881.
Kim, S.J., Nara, E., Kobayashi, H., Terao, J., Nagao, A., 2001. Formation of cleavage products by autoxidation of lycopene. Lipids 36, 191–199.
Kim, L., Rao, V., Rao, L., 2002. Effects of Lycopene on prostate LNCaP cancer cells in culture. J. Med. Food 5, 181–187.
Kopec, R.E., Riedl, K.M., Harrison, E.H., Curley Jr., R.W., Hruszkewycz, D.P., Clinton, S.K., Schwartz, S.J., 2010. Identification and quantification of apolycopenals in fruits, vegetables, and human plasma. J. Agric. Food Chem. 58, 3290–3296.
Kotake-Nara, E., Kushiro, M., Zhang, H., Sugawara, T., Miyashita, K., Nagao, A., 2001. Carotenoids affect proliferation of human prostate Cancer cells. J. Nutr. 131, 3303–3306.
Lakshminarayana, R., Aruna, G., Sangeetha, R.K., Bhaskar, N., Divakar, S., Baskaran, V., 2008. Possible degradation/biotransformation of lutein in vitro and in vivo: isolation and structural elucidation of lutein metabolites by HPLC and LC–MS (atmospheric pressure chemical ionization). Free Radic. Biol. Med. 45, 982–993.
Lakshminarayana, R., Sathish, V., Shylaja, M.D., Baskaran, V., 2010. Antioxidant and cytotoxic effect of oxidized lutein in human carcinoma cells (HeLa). Food Chem. Toxicol. 48, 1811–1816.
Lakshminarayana, R., Aruna, G., Sathisha, U.V., Dharmesh, S.M., Baskaran, V., 2013. Structural elucidation of possible lutein oxidation products mediated through peroxyl radical inducer 2, 2′-Azobis (2-methylpropionamidine) dihydrochloride: antioxidant and cytotoxic influence of oxidized lutein in HeLa cells. Chem. Biol. Inter 203, 448–455.
Liu, C., Lian, F., Smith, D.E., Russell, R.M., Wang, X.D., 2003. Lycopene supplementation inhibits lung squamous metaplasia and induces apoptosis via up-regulating insulin-like growth factor-binding protein 3 in cigarette smoke-exposed ferrets. Cancer Res. 63, 3138–3144.
Livny, O., Kaplan, I., Reifen, R., Polak-Charcon, S., Madar, Z., Schwartz, B., 2002. Lycopene inhibits proliferation and enhances gap-junction communication of KB-1 human oral tumor cells. J. Nutr. 132, 3754–3759.
Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein estimation with Folin phenol reagent. J. Biol. Chem. 193, 265–275.
Nahum, A., Hirsch, K., Danilenko, M., Watts, C.K.W., Prall, O.W.J., Levy, Y., Sharoni, Y., 2001. Lycopene inhibition of cell cycle progression in breast and endometrial cancer cells is associated with reduction in cyclin D levels and retention of p27Kip1 in the cyclin E-cdk2 complexes. Oncogene 20, 3428–3436.
Nara, E., Hayashi, H., Kotake, M., Miyashita, K., Nagao, A., 2001. Acyclic carotenoids and their oxidation mixtures inhibit the growth of HL-60 human promyelocytic leukemia cells. Nutr. Cancer 39, 273–283.
Nidhi, B., Sharavana, G., Ramaprasad, T.R., Basakaran, V., 2015. Lutein derived fragments exhibit higher antioxidant and anti-inflammatory properties than lutein in lipopolysaccharide induced inflammation in rats. Food Funct. 6, 450–460.
Ohkawa, H., Ohishi, N., Yagi, H., 1979. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal. Biochem. 95, 351–358.
Omenn, G.S., Goodman, G.E., Thomquist, M.D., 1996. Effects of a combination of β-carotene and vitamin A on lung cancer and cardiovascular disease. New Engl. J. Med. 334, 1150–1155.
Palozza, P., Calviello, G., Serini, S., Maggiano, N., Lanza, P., Ranelletti, F.O., Bartoli, G.M., 2001. Beta-carotene at high concentrations induces apoptosis by enhancing oxy-radical production in human adenocarcinoma cells. Free Radic. Biol. Med. 30, 1000–1007.
Palozza, P., Serini, S., Maggiano, N., Angelini, M., Boninsegna, A., Di Nicuolo, F., Ranelletti, F.O., Calviello, G., 2002. Induction of cell cycle arrest and apoptosis in human colon adenocarcinoma cell lines by β-carotene through down-regulation of cyclin A and Bcl-2 family proteins. Carcinogenesis 23, 11–18.
Pennathur, S., Maitra, D., Byun, J., Sliskovic, I., Abdul Hamid, I., Saed, G.M., Diamond, M.P., Abu-Soud, H.M., 2010. Potent antioxidative activity of lycopene: a potential role in scavenging hypochlorous acid. Free Radic. Biol. Med. 49, 205–213.
Prasad, V., Chandele, A., Jagtap, J.C., Sudheer, K.P., Shastry, P., 2006. ROS-triggered caspase 2 activation and feedback amplification loop in beta-carotene-induced apoptosis. Free Radic. Biol. Med. 41, 431–442.
Rao, A.V., Agarwal, S., 2000. Role of antioxidant lycopene in cancer and heart disease. J. Am. Coll. Nutr. 19, 563–569.
Rodriguez, E.B., Rodriguez-Amaya, D.B., 2009. Lycopene epoxides and apolycopenals formed by chemical reactions and autoxidation in model systems and processed foods. J. Food Sci. 74, 674–682.
Sharoni, Y., Linnewiel-Hermoni, K., Zango, G., Khanin, M., Salman, H., Veprik, A., Danilenko, M., Levy, J.M., Levy, J., 2012. The role of lycopene and its derivatives in the regulation of transcription systems: implication for cancer prevention. Am. J. Clin. Nutr. 96, 1173S–1178S.
Siems, W., Sommerburg, O., Schild, L., Augustin, W., Langhans, C.D., Wiswedel, I., 2002. β-carotene cleavage products induce oxidative stress in vitro by impairing mitochondrial respiration. FASEB J. 16, 1289–1291.
Soares, N.C.P., Teodoro, A.J., Oliveira, F.L., Takiya, C.M., Junior, A.P., Nasciutti, L.E., Lotsch, P.F., Granjeiro, J.M., Ferreira, L.B., Gimba, E.R.P., Borojevic, R., 2014. Lycopene induce apoptosis in human prostate cells and alters the expression of Bax and Bcl-2 genes. LWT - Food Sci. Tech. 59, 1290–1297.
Sowmya, P.R., Arathi, B.P., Vijay, K., Baskaran, V., Lakshminarayana, R., 2014. Optimization of LC/MS (APCI) $^{+ve}$ methods for the determination of possible lutein oxidation products in plasma and tissues of adult rats. Chromatographia 77, 1633–1642.
Sowmya, P.R., Arathi, B.P., Vijay, K., Baskaran, V., Lakshminarayana, R., 2015. Role of different vehicles in carotenoids delivery and their influence on cell viability, cell cycle progression, and induction of apoptosis in HeLa cells. Mol. Cell. Biochem. 406, 245–253.
Talvas, J., Veyrat, C.C., Guy, L., Rambeau, M., Lyan, B., Quinard, R.M., 2010. Differential effects of lycopene consumed in tomato paste and lycopene in the form of a purified extract on target genes of cancer prostatic cells. Am. J. Clin. Nutr. 91, 1716–1724.
Tang, L., Jin, T., Zeng, X., Wang, J.S., 2005. Lycopene inhibits the growth of human androgen-independent prostate cancer cells in vitro and in BALB/c nude mice. J. Nutr. 135, 287–290.
Teodoro, A.J., Oliveira, F.L., Martins, N.B., Maia, G.D.A., Martucci, R.B., Borojevic, R., 2012. Effect of lycopene on cell viability and cell cycle progression in human cancer cell lines. Cancer Cell Int. 12, 36.
Tietze, F., 1969. Enzymatic method for quantitative determination of nano gram amounts of total and oxidized glutathione: applications to mammalian blood and other tissues. Anal. Biochem. 27, 502–522.
Wang, Xiang-Dong, 2012. Lycopene metabolism and its biological significance. Am. J. Clin. Nutr. 96, 1214S–1222S.
Wang, Xiang-Dong, Robert, M.R., 1999. Procarcinogenic and anticarcinogenic effects of β-carotene. Nutr. Rev. 57, 263–272.
Zhang, H., Kotake-Nara, E., Ono, H., Nagao, A., 2003. A novel cleavage product formed by autoxidation of lycopene induces apoptosis in HL-60 cells. Free Radic. Biol. Med. 35, 1653–1663.